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Occurrence of mycotoxins in extruded commercial dog food

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Abbreviations: AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; BW, body weight; DM, dry matter; DON, deoxynivalenol; FB_1, fumonisin B_1; FB_2, fumonisin B_2; LC-MS, liquid chromatography coupled to mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; OTA, ochratoxin A; RO, Reverse Osmosis; UP, Ultra Pure; UPLC-MS/MS, ultra-performance liquid chromatography coupled to tandem mass spectrometry; ZEA, zearalenone.

Abstract

The aim of this study was to determine the presence and the level of contamination of the most important mycotoxins (deoxynivalenol, fumonisin B_1 and B_2, aflatoxin B_1, B_2, G_1 and G_2, ochratoxin A and zearalenone) in 48 samples of extruded dry dog food found in the Italian market (24 samples from standard economy lines, 24 of premium lines). Analyses were performed using ultra-performance liquid chromatography coupled to tandem mass spectrometry. Although the concentrations of the mycotoxins in all samples proved to respect the European legislation with regards to animal feed, the analyses revealed a substantial presence of deoxynivalenol, fumonisins and ochratoxin A, with values above the limit of quantification (5 µg/kg) in 100%, 88% and 81% of the samples, respectively. In contrast, aflatoxins and zearalenone contamination proved to be very modest, with 88% and 75% of the samples, respectively, showing concentrations below the corresponding limit of quantification (5 µg/kg for aflatoxins and 10 µg/kg for zearalenone). Moreover, despite a very heterogeneous contamination, the concentration of fumonisins and ochratoxin A was significantly higher in standard foods than in premium ones (491 *vs.* 80.2 µg/kg dry matter for fumonisin B_1; 113 *vs.* 38.5 µg/kg dry matter for fumonisin B_2; 599 *vs.* 103 µg/kg dry matter for total fumonisins; 23.8 *vs.* 13.0 µg/kg dry matter for ochratoxin A; $P < 0.001$). Furthermore, a simultaneous presence of different mycotoxins (at concentrations higher than their limit of quantification) was observed in most of the pet foods analyzed; in particular, 19% of the samples were contaminated by no fewer than two different types of mycotoxins, 52% by three, 25% by four and 2% by all the mycotoxins evaluated. These results revealed the need for further investigation into the potential risk deriving from chronic exposure to low doses of the different types of mycotoxins that pet species are subject to today.

Keywords: dog foods, mycotoxins, ultra-performance liquid chromatography, mass spectrometry.

1. Introduction

Food quality and safety have presently gained considerable importance in the public opinion. In the veterinary field the need to ensure the safety of products of animal origin is reflected nowadays by the routine of performing rigorous tests on feeds intended for livestock animal species, as the foods derived from them represent potential vehicles of substances that are hazardous to humans (EC, 2004).

In consideration of the recent strengthening of the human-pet bond and increased health awareness, as well as the more general concern for pet welfare (Walsh, 2009), the issue of pet food quality and safety is significantly impacting the pet food industry, which today plays a role of considerable importance insofar as the nutritional management of pets is concerned (Assalco, 2014). In this area, mycotoxin contamination, in particular, is drawing increasing interest.

The traditional use of a large quantity of vegetable ingredients and by-products (cereals, for example) by pet food manufacturers, particularly in the formulations of dry products, has enormously favored the risk of mycotoxin intoxication in pet species (Leung et al., 2006; Boermans and Leung, 2007), given that the various steps of the pet food production process are not able to completely inactivate these fungal metabolites (Bullerman and Bianchini, 2007).

In the recent past, some monitoring initiatives carried out in different parts of the world have revealed a significant presence of mycotoxins in the pet food samples analyzed. More specifically, the principal mycotoxins investigated were aflatoxins, fumonisins, deoxynivalenol (DON), zearalenone (ZEA) and ochratoxin A (OTA) (Leung et al., 2006; Boermans and Leung, 2007; Songsermsakul et al., 2007; Böhm et al., 2010; Pagliuca et al., 2011).

With regard to the legislative and regulatory sphere, the situation on an international level is still not sufficiently defined and harmonized. In fact, the reference provisions are mostly aimed at food and feed intended for humans and livestock animals, rather than pet species, with ample variability in terms of tolerance limits among the numerous countries concerned (Mazumder and Sasmal, 2001; EC, 2002; FAO, 2004; EC, 2006; van Egmond et al., 2007).

Although the knowledge about the toxicological effects of mycotoxins in dogs and cats is still limited, some studies have shown that the presence of such substances in pet food can cause serious harm to pet health, with both acute and chronic forms of intoxication depending on the level of contamination and length of exposure (Leung et al., 2006; Boermans and Leung, 2007; Newman et al., 2007; Dereszynski et al., 2008; Bruchim et al., 2012; Wouters et al., 2013).

This study was aimed at identifying and quantifying the main mycotoxins considered under European legislation in complete industrial dry dog foods available in the Italian market and belonging to different price ranges.

2. Materials and methods

2.1. Sampling

Forty-eight complete commercial extruded dry dog foods were purchased from stores in the province of Bologna (Italy). Specifically, the products included 24 low/standard dog foods (consisting in economical formulations ranging in price from € 0.80 to 4.00/kg, sold by discount and mass-market retailers) and 24 premium/super premium dog foods (consisting in more costly formulations ranging in prices from € 4.00 to 15.00/kg, found in specialized stores). The size of the packages purchased was in the range of 300 g to 5 kg.

Particularly, this reference species was chosen for the study, as dog foods generally contain larger quantities of cereal ingredients than those formulated for the feline species, and thus dogs are likely to be exposed to a greater risk of contamination than cats.

All the analyses were conducted on a representative sample of each product (about half of the content of every package was ground and used for chemical analyses and mycotoxins determination).

2.2. Chemical analyses of the samples

The pet food samples were subjected to chemical analysis to determine moisture and starch content according to the official methods of the Association of Official Analytical Chemists (AOAC, 2000; method 950.46 for moisture and method 996.11 for starch).

2.3. Determination of mycotoxin concentration

Chemicals and reagents. Aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), DON, ZEA and OTA standards were purchased from Sigma-Aldrich (Steinheim, Germany).

U-[$^{13}C_{17}$]-AFB_1 , U-[$^{13}C_{34}$]-FB_1, U-[$^{13}C_{15}$]-DON, U-[$^{13}C_{18}$]-ZEA and U-[$^{13}C_{20}$]-OTA were obtained from Romer Lab Inc-Biopure (Tulln, Austria).

Methanol and formic acid, used as the mobile phases, and ammonium acetate were of analytical grade specific for liquid chromatography coupled to mass spectrometry (LC-MS) analysis and were purchased from Riedel-de Haën (Seelze, Germany). Acetonitrile and acetic acid, used in the extraction procedures, were purchased from Merck (Darmstadt, Germany).

Reverse Osmosis (RO) and Ultra Pure (UP) water, respectively used as an extraction solvent and mobile phase, were produced by a Human Power apparatus from Human Corporation (Seul, Korea).

Sample preparation. One gram of ground sample was weighed into a beaker, fortified with labeled standards and extracted with 4 mL of acetonitrile:water:acetic acid solution. The sample was shaken for 2 hours using the orbital shaker and was then centrifuged. 500 µL of the supernatant was collected and dried under a nitrogen stream at 40 °C. At the end, the sample was redissolved in 500 µL of a mixture of water:acetonitrile:formic acid with ammonium acetate and filtered. 10 µL of sample extract was analyzed using the ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS).

UPLC-MS/MS equipment and conditions. The analysis was realized by UPLC-MS/MS, composed of a Waters Acquity UPLC binary pump, equipped with a Waters Acquity BEH C_{18} reversed-phase column coupled to a VanGuard guard column with identical packaging (Waters, Milford, MA, USA).

Water containing 0.1% formic acid (solvent A) and methanol containing 0.1% formic acid (solvent B) were employed as mobile phases under programmed conditions at a flow rate of 0.42 mL/min. The analysis was carried out over 16 min using a previous method developed by Jackson et al. (2012). The column heater temperature was set at 40 °C and the volume injection was 10 µL.

The mass spectrometer was a Quattro Premier XE, a triple quadrupole instrument equipped with an ESCI™ Multi-Mode Ionization Source (Waters, Milford, MA, USA).

The mass spectrometer operated in the positive electrospray ionization mode (ESI+) using multiple reaction monitoring (MRM). The capillary voltage was set at 3.5 kV; the MRM transitions, cone voltages and collision energies are reported in Table 1.

Data acquisition and processing was performed using Mass Lynx 4.1 Software (Waters Corporation, Milford, USA).

160

161 2.4. Statistical analyses

162 The concentrations of the different mycotoxins detected in the standard and premium pet
163 foods were subjected to statistical analysis of variance using Student's t-test in order to
164 determine any statistically significant differences between the two price categories considered.
165 For the purposes of statistical analysis, samples in which it was not possible to detect or quantify
166 the concentration of given mycotoxins were assigned accordingly to the corresponding
167 reference limit of detection (LOD) or limit of quantification (LOQ). Differences were
168 considered statistically significant when $P < 0.05$.

169

170 **3. Results**

171 Chemical analysis of the pet food samples revealed a practically equivalent mean moisture
172 and starch content in the two pet food categories considered; in fact, the water concentration
173 was 71±11 and 61±11 g/kg, while the starch content was 362±54 and 363±66 g/kg, in the
174 standard and premium foods, respectively.

175 The data relating to positivity for and concentrations of the different mycotoxins in the two
176 pet food categories are illustrated in Tables 2 and 3, respectively.

177 DON was quantified in all samples analyzed (it was thus always present in a concentration
178 ≥ the LOQ, 5 µg/kg), with no significant difference between standard and premium products.

179 With regard to aflatoxins, the analyses carried out revealed the presence of these
180 contaminants in trace amounts, which were below the limit of quantification in 75% of the
181 samples examined; none of the analyzed samples contained AFB_1 and AFG_1 at levels above the
182 LOQ (0.5 µg/kg) while measurable, albeit very modest, concentrations of AFB_2 or AFG_2 were
183 detected in only 6 samples (Table 2) (with concentrations between 5.7 and 15.8 µg/kg dry
184 matter (DM)).

Concerning fumonisins, the results obtained showed a broad range of contamination, with significantly higher values in standard products than in premium ones, as regards both the individual fumonisins considered (B_1 and B_2) and total fumonisins ($B_1 + B_2$) ($P < 0.001$) (Table 3). Moreover, this category of mycotoxins was detected or quantified in all samples (with the exception of FB_2 which was not identified in 8 out of 48 samples) (Table 2).

OTA was found in quantities between the LOD (2 µg/kg) and the LOQ (5 µg/kg) and exceeding the LOQ, respectively, in 17% and 81% of the samples analyzed (Table 2), with values significantly higher in standard products than in premium ones ($P < 0.001$) (Table 3).

ZEA was found to be present at rather low levels in both commercial pet food categories considered; trace amounts were detected in 35% of the total samples and a modest concentration was quantified in 25% of them (Table 2), with values falling in a particularly narrow range (10.4 and 42.4 µg/kg DM).

The results achieved through this study showed that all standard samples and most of the premium samples (23 out of 24) contained quantified amounts of, at least, two types of mycotoxins. In particular, over half of the pet food samples analyzed (52%; 15 standard and 10 premium samples) showed to be contaminated by three different types of mycotoxins, 25% (6 standard and 6 premium samples) by four, 19% (3 standard and 6 premium) by two and 2% (1 premium sample) by the five mycotoxin categories evaluated (Table 4). In particular, a high number of samples presented the co-occurrence of *Fusarium* mycotoxins: in fact, 22 out of 24 standard samples and 20 out of 24 premium samples presented the co-occurrence of DON and fumonisins (FB_1 and/or FB_2). Among these samples, 4 standard and 4 premium samples were contaminated also by ZEA.

4. Discussion

209 The problem of mycotoxin contamination has represented for a long time a great concern for
210 feed and food industries, not only for the dangerous consequences on human health, but also
211 for the relevant economic implications associated to agricultural and zootechnical losses.
212 Amongst recent concerns about the quality and safety of pet foods, the occurrence of
213 mycotoxins represents a serious problem for pet health, with both economic and emotional
214 implications for owners.
215 In the European Union, AFB_1 is the only mycotoxin for which precise maximum limits have
216 been established for complete and complementary feedingstuffs intended for animals (under
217 Directive 2002/32/EC and subsequent amendments: 5-20 µg/kg with reference to products
218 containing 12% moisture), with several specifications for farm animals. With regard to the
219 presence of other types of mycotoxins in products intended for animal feeding, considered in
220 European legislation (DON, T-2 and HT-2 toxins, $FB_1 + FB_2$, OTA and ZEA), there are simple
221 "guidance values" (EC, 2006 and EC, 2013). The recommendations specify some
222 differentiations based on the type of vegetable raw materials considered or, in the case of
223 complete or complementary feed containing 12% moisture, the animal species and the
224 production type for which they are intended. However, there are no specific guidelines relating
225 to pets, with the sole exception of the category of total fumonisins ($B_1 + B_2$) and *Fusarium*-
226 toxins T-2 and HT-2, for which precise guidance values are specified (5000 µg/kg for
227 fumonisins in foods for pet animals and 50 µg/kg for T-2 and HT-2 toxins in foods for cats).
228 The results of the present monitoring showed that none of the pet food samples tested
229 contained concentrations of mycotoxins exceeding the limits specified in the above-mentioned
230 rules. However, it was possible to observe, in general, a noteworthy presence of two mycotoxins
231 produced by moulds of the genus *Fusarium*, namely, DON and fumonisins, and of a mycotoxin
232 produced by moulds belonging to the genera *Aspergillus* and *Penicillium*, i.e. OTA.

More specifically, DON was the only mycotoxin quantified in all samples analyzed (Table 2). Several toxicological studies conducted on pet species have shown that loss of appetite and vomiting are the main symptoms caused by this mycotoxin in doses equal to or greater than 3 and 10 mg/kg of food, respectively, in dogs and cats (Hughes et al., 1999; Leung et al., 2007); these values far exceed those found in the present study.

The high incidence of DON in dry pet food has also been confirmed by previous studies conducted in Austria. In the course of an investigation conducted on complete foods intended for the canine species, Songsermsakul et al. (2007) found DON contamination in all dry food samples, with a rather broad range of between 22 and 1837 µg/kg, and in 27% of wet foods, though in this case the concentrations were lower on average and fell within a narrower range (95-170 µg/kg). The widespread presence of DON in dog food has been highlighted more recently also by Böhm et al. (2010) in a study, similarly carried out in Austria, in which contamination by this mycotoxin was found in 83% of the dry pet food samples tested, with a mean and maximum concentration of 308 and 1390 µg/kg, respectively. Again, these values far exceed those observed in the present study.

Fumonisins were found to be present in all samples analyzed and it was possible to quantify them in 88% of the cases (i.e. in 42 out of 48 samples). The toxicity of these mycotoxins is essentially associated with an alteration in the metabolism of cellular sphingolipids and the consequent activation of mechanisms of apoptosis, necrosis and compensatory hyperplasia (Wang et al., 1991). Although we presently have scant knowledge about the effects of fumonisins in dogs and cats, several studies on other animal species have shown them to be capable of determining phenomena of hepato- and nephrotoxicity in cases of acute intoxication and immunodepression in those of a chronic nature (Boermans and Leung, 2007), with a species specificity that is generally high insofar as the clinical manifestations are concerned: for example, leukoencephalomalacia in equidae (Dutton, 1996), lethal hepatic and renal lesions in

rats and rabbits (Voss et al., 1998; Voss et al., 2001), and hepatic necrosis and pulmonary edema in pigs (Placinta et al., 1999).

The fumonisins contamination found in the present study was higher than that observed by other authors in the course of analogous studies conducted in Europe, in terms of both the mean and maximum concentrations detected. Martins et al. (2003) found FB_1 to be present in only 5% of the tested samples of pet foods sold in the Portuguese market, with contamination ranging between 12 and 24 µg/kg. In the previously cited study conducted in Austria by Böhm et al. (2010), the level of fumonisins contamination was likewise rather modest, despite being found in a more significant percentage (42%) of the pet food samples analyzed, with mean and maximum total fumonisins concentrations of 122 µg/kg and 568 µg/kg, respectively. As already pointed out, these values are decidedly lower than the ones revealed in the present study and this is particularly true in the case of standard products, in which mean and maximum levels of contamination of 491 and 1503 µg/kg DM, respectively, were observed in the case of FB_1. Recently, an Italian study which screened samples of dry dog foods, both complete and complementary, revealed widespread fumonisins contamination in all samples analyzed, with higher levels on average in products belonging to the lower price range and even two cases of products exceeding the tolerance limits set by current European legislation (Pagliuca et al., 2011).

Another *Fusarium* mycotoxin undergoing assessment, ZEA, showed a rather limited level of contamination in all samples, with quantifiable concentrations in only 25% of them. In this regard, it should be noted that in the analytical method used for ZEA, the LOQ was higher than that applied for the other mycotoxins (10 *vs.* 5 µg/kg).

The toxicity of ZEA, well known for its estrogenic and anabolic effects - which have repercussions mainly for the reproductive system (Boermans and Leung, 2007) - has also been investigated in pets. In particular, some by now outdated studies demonstrated the toxicological

283 effects of this mycotoxin in dogs with daily dosages of 5 mg/kg of body weight (BW) after a
284 period of intake of 13 weeks (Hidy et al., 1977). Other more recent studies have highlighted
285 that alterations in the dog's reproductive system are already evident after 7 days at daily doses
286 of 200 µg/kg of BW (Gajecka et al., 2004) - much smaller doses than in the previous study -
287 and that appreciable effects on the blood concentrations of sexual hormones could also be
288 observed following exposure to smaller doses for a longer period of time (75 µg/kg of BW for
289 42 days) (Gajecka et al., 2013).
290 Unlike the present study, the survey conducted in Austria by Böhm et al. (2010) revealed a
291 widespread and substantial ZEA contamination; in fact, 47% of the dog food samples tested
292 were positive, with a mean and maximum concentration of 51 and 298 µg/kg, respectively. A
293 similar heterogeneity of contamination among positive samples and a rather high level of
294 contamination had also emerged during a previous study conducted in Poland by
295 Zwierzchowski et al. (2004); the presence of ZEA was observed in no less than 84% of the
296 tested dry dog foods, which belonged to different price ranges.
297 Concerning aflatoxins, their presence in the samples examined was very modest, with
298 quantifiable concentrations in only 6 out of 48 samples.
299 Aflatoxins are well known for their hepatotoxic and carcinogenic effects in all animal species
300 studied; these effects may be both acute and chronic, depending on the level of exposure
301 (Boermans and Leung, 2007). In particular, the dog appears to be one of the domestic species
302 most sensitive to the effects deriving from intoxication by aflatoxins, most likely because of the
303 low activity of glutathione S-transferase, which is involved in the detoxification of these
304 mycotoxins (Watanabe et al., 2004). In acute forms of aflatoxicosis, dogs exposed to doses
305 exceeding 500-1000 µg/kg of BW died within a few days, showing signs of hepatic hyperplasia,
306 disseminated intravascular coagulation and hemorrhages. Furthermore, whereas subacute forms
307 - observed following the intake of foods contaminated by aflatoxins at concentrations of around

500-1000 µg/kg - are typically characterized by anorexia, lethargy, jaundice, disseminated intravascular coagulation and death after 2-3 weeks, in chronic forms the same clinical symptoms are associated with an exposure to concentrations of 50-300 µg/kg of food for 6-8 weeks (Böhm and Razzazi-Fazeli, 2005). A relevant case of canine aflatoxicosis, which occurred in the United States between 2005 and 2006 due to the intake of a commercial dry product contaminated with AFB_1 in a range of 223 to 579 µg/kg of food, provoked the death of nearly all the intoxicated animals as a result of a severe form of liver failure (Newman et al., 2007).

Various investigations conducted on dog food samples in South America and Turkey in the past decade have revealed a significant presence of aflatoxins in these products, with concentrations sometimes exceeding the maximum limits allowed by the respective national legislation (Sharma and Marquez, 2001; Maia and Pereira Bastos de Siqueira, 2002; Gunsen and Yaroglu, 2003; Scussel et al., 2006). In contrast, other studies recently conducted in several European countries showed only a modest presence of aflatoxins in dog foods, often in non-quantifiable traces or, in any case, in very small concentrations (Martins et al., 2003; Lopez-Grio et al., 2010; Böhm et al., 2010). This situation, confirmed by the results of the present study, is likely to be attributable to the effectiveness of the control system currently in force in Europe, particularly with respect to aflatoxins in feed intended for animals. As pointed out earlier, AFB_1 currently is the only mycotoxin for which European legislation has fixed precise maximum limits of tolerated contamination (EC, 2002). Not coincidentally, given that no specific guidelines for pet foods exist at present, the notifications recorded in the database of the European Rapid Alert System for Food and Feed (RASFF) about pet foods - nearly all of which concern border rejection of vegetable raw materials imported from non-EU countries - regard the presence of aflatoxins (EC, RASFF Portal).

With regard to OTA, it was quantified in 81% of the samples analyzed. The dog appears to be a species that is particularly vulnerable to this mycotoxin, which is well known for its nephrotoxic and immunosuppressive effects (Duarte et al., 2010). Several studies conducted in the past on Beagles showed that OTA was lethal in daily doses of 200 µg/kg of BW for 2 weeks or in a single dose of 7.8 mg/kg of BW (Szczech et al., 1973). Symptoms such as anorexia, weight loss, vomiting, tenesmus, hemorrhagic diarrhea, dehydration and prostration have also been observed in dogs that had been exposed to this mycotoxin, again for 2 weeks, in doses of between 0.2 and 30 mg/kg of BW (Kitchen et al., 1977).

Unlike the other monitored mycotoxins, OTA is a contaminant commonly found not only in vegetable foods, but also in matrices of animal origin, as a result of the accumulation of these compounds in muscles, organs and offal (kidneys and liver, in particular), which are often used in high quantities by the pet food industry, especially for the formulation of wet products (Mantrella et al., 2006; Pfohl-Leszkowicz and Manderville, 2007). For this reason, the studies available in the literature regarding OTA in pet food also consider wet pet food. Razzazi et al. (2001), for example, quantified this mycotoxin in 60% of pet food samples (a total of 10 dry and 30 wet foods, respectively) purchased in the Polish and Austrian markets, with analogous percentages of positivity in the two types of products (respectively 40% and 43%), albeit with different levels of contamination (in the range of 0.21-13.1 µg/kg and 0.22-0.8 µg/kg, in dry and wet pet foods, respectively). Other studies conducted in Europe have shown, in contrast, a more sporadic OTA contamination in the pet food samples examined, for the most part in rather modest concentrations, always lower than 5 µg/kg (Martins et al., 2003; Lopez-Grio et al., 2010; Böhm et al., 2010). In a study conducted on 40 dry and wet dog foods available in the Austrian and German markets, Songsermsakul et al. (2007) observed a range of OTA contamination (from 7 to 40 µg/kg) similar to the one found in the present study, though this mycotoxin was quantifiable in only 12.5% of the foods.

Although the concentrations of mycotoxins detected in the present study were always well below regulatory limits and the levels of contamination associated with cases of acute and subacute mycotoxicosis in pets (Hughes et al., 1999; Stenske et al., 2006; Newman et al., 2007; Leung et al., 2007), our investigation confirms the problem of "multiresidues", recently pointed out also in feedingstuffs intended for farm animals (Zachariasova et al., 2014). Mycotoxin co-occurrence in pet food was previously highlighted by other European authors (Böhm et al., 2010) and seems to concern especially *Fusarium* mycotoxins (fumonisins and DON, in particular). In fact, also in the present study, a significant number of samples (22 out of 24 standard samples and 20 out of 24 premium samples) presented the co-occurrence of DON and fumonisins (FB_1 and/or FB_2), often in association with ZEA and/or OTA.

It needs to be stressed that the problem of mycotoxin co-occurrence not only may concern the finished products (such as pet food) but also the single ingredients (cereals in particular). These vegetable ingredients, corn in particular, appear to pose a strong risk of multi-contamination, as attested by numerous studies conducted both in Europe and South America (Streit et al., 2012). The toxicological effects deriving from the interaction between different mycotoxins (mainly of the additive or synergistic type) have been investigated both on laboratory and livestock animals. These studies have revealed a rather complex situation, which is strongly dependent on numerous factors such as the animal species, the mycotoxin type and dose, as well as the duration of exposure and type of monitored parameters (Grenier and Osvald, 2011).

It has been observed, for example, that the simultaneous presence of OTA and fumonisins in feeds intended for pigs favors a "multi-toxicological" effect, with sero-hemorrhagic ascites and pulmonary hemorrhages and hydrothorax among the principal symptoms (Stoev et al., 2010). Moreover, synergistic effects on both liver and kidneys were observed after the ingestion of feed by rabbits contaminated with AFB_1 and FB_1 (Orsi et al., 2007). Furthermore, several in

382 vitro investigations conducted on different human and pig cell lines seem to have confirmed an
383 increase in cytotoxicity associated with the simultaneous presence of OTA and FB_1 (Creppy et
384 al., 2004; Mwanza et al., 2009).

385 It has recently been hypothesized that the canine aflatoxicosis outbreak occurring in South
386 Africa in 2011 may have had a multi-mycotoxicological etiology. An analysis conducted on 60
387 samples of dog foods sold in that country in the period concerned not only revealed a high level
388 of positivity for aflatoxins, with concentrations above the limit prescribed by law in 75% of the
389 positive samples, but also showed a substantial number of other mycotoxins, such as
390 fumonisins, OTA and ZEA, at concentrations considerably exceeding the tolerable limits set up
391 by the current legislations. This situation is likely to have favored additive and/or synergistic
392 interactions among the different mycotoxins, which may have influenced the clinical expression
393 of this recent phenomenon of mycotoxicosis in the canine species (Mwanza et al., 2013).

394 Information about the synergistic effects deriving from the long-term intake of foods
395 contaminated with low concentrations of mycotoxins in pet species is completely lacking at
396 present. In consideration of the ample evidence reported in the literature, it would therefore be
397 desirable to gain further knowledge on this subject and update legislative provisions
398 accordingly in order to provide useful references for addressing the frequent cases of multi-
399 contamination in feeds intended for different animal species.

400 The present investigation revealed a higher presence of fumonisins (B_1, B_2 and total) and
401 OTA in lower-priced dog food products. Because the average starch content in standard foods
402 and higher priced ones showed to be nearly identical, the higher presence of mycotoxins that
403 was observed in lower-priced samples is likely to be the consequence of a lower quality of the
404 cereals used rather than larger inclusion levels of these raw materials.

405 In Europe, cereal foodstuffs, ingredients widely present in dry pet food formulations, are
406 universally affected by mycotoxin contamination. This situation is difficult to predict and

control since it is influenced by numerous factors, such as climatic conditions, which render the entity of contamination extremely variable from one year to the next. Moreover, methods used to detect and quantify the different molecules and the sampling procedure are principal critical aspects from a sampling and analytical point of view (Streit et al., 2012). In particular, the sampling step usually represents the largest source of error due to the extreme heterogeneous mycotoxin distribution in feedstuffs. In fact, it is difficult to obtain accurate estimates of the true mycotoxin concentration of a bulk lot when using a sampling plan that is not the accumulation of many small incremental portions taken at many different locations throughout the lot (Whitaker, 2003).

At present, manufacturers are not obliged to provide detailed information on the packages of dog and cat foods as to the type and quantity of the different vegetable sources used, so that it is not possible to associate the greater presence of given mycotoxins to the use of specific raw materials, for example OTA in barley and fumonisins in corn, as reported by Bennet and Clich (2003). However, the lower concentrations of fumonisins and OTA detected in premium pet foods are most likely attributable to a higher quality level of the raw materials and more attentive controls over the ingredients used, thanks to the use of modern technologies presently available to industry operators (Mabbet, 2014).

5. Conclusions

The present study has shown that all the samples of complete extruded dog foods considered complied with current European legislation regarding mycotoxin contamination. Notwithstanding this, the widespread presence, in all pet foods analyzed, of multiple types of mycotoxins (mainly of *Fusarium* mycotoxins and OTA), though individually present in modest concentrations, underscores the need to further investigate the potential synergistic effects that

could occur given this situation. Moreover, foods falling within the standard price range were more polluted on average by the presence of fumonisins and OTA than higher priced foods.

In consideration of the chronic exposure to which a pet is potentially exposed when it receives the same contaminated food for a long period of time, we can perceive the advisability of incentivizing pet food manufacturers to test for mycotoxins. This may also be achieved by improving the provisions of law where necessary.

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601

602 **Table 1**

603 Mass spectrometry parameters of selected mycotoxins.

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Cone Voltage (kV)	Collision Energy (eV)
DON	296.90	230.90	19	12
		248.90	19	10
AFB_1	312.80	241.00	45	36
		285.00	45	22
AFB_2	315.00	259.00	45	38
		287.00	45	33
AFG_1	329.00	243.00	45	26
		283.00	45	24
AFG_2	331.00	245.00	46	39
		313.00	46	33
FB_1	722.20	334.20	52	45
		352.20	52	43
FB_2	706.10	318.30	50	40
		336.34	50	38
OTA	403.90	221.90	25	37
		238.90	25	25
ZEA	318.95	184.90	20	30
		282.90	20	12

604 DON, deoxynivalenol; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2,

605 aflatoxin G_2; FB_1, fumonisin B_1; FB_2, fumonisin B_2; OTA, ochratoxin A; ZEA, zearalenone.

606

Table 2

Positivity for mycotoxins of commercial extruded dry dog food.

	Number of positive samples					
	LOD[a] < mycotoxin > LOQ[b]			Mycotoxin ≥ LOQ[b]		
Mycotoxin	Standard (n=24)	Premium (n=24)	Total (n=48)	Standard (n=24)	Premium (n=24)	Total (n=48)
DON	0	0	0	24 (100%)	24 (100%)	48 (100%)
AFB_1	11 (46%)	5 (21%)	16 (33%)	0	0	0
AFB_2	11 (46%)	15 (63%)	26 (54%)	0	2 (8%)	2 (4%)
AFG_1	1 (4.2%)	7 (29%)	8 (17%)	0	0	0
AFG_2	11 (46%)	7 (29%)	18 (38%)	2 (8%)	2 (8%)	4 (8%)
Aflatoxins[c]	19 (79%)	17 (71%)	36 (75%)	2 (8%)	4 (17%)	6 (12%)
FB_1	2 (8%)	5 (21%)	7 (15%)	22 (92%)	19 (79%)	41 (85%)
FB_2	1 (4%)	8 (33%)	9 (19%)	21 (88%)	14 (58%)	35 (73%)
Fumonisins[d]	2 (8%)	4 (17%)	6 (12%)	22 (92%)	20 (83%)	42 (88%)
OTA	1 (4%)	7 (29%)	8 (17%)	22 (92%)	17 (71%)	39 (81%)
ZEA	6 (25%)	11 (46%)	17 (35%)	5 (21%)	7 (29%)	12 (25%)

DON, deoxynivalenol; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; FB_1, fumonisin B_1; FB_2, fumonisin B_2; OTA, ochratoxin A; ZEA, zearalenone.

[a] LOD: limit of detection (DON: 1 µg/Kg; AFB_1 and AFB_2: 0.5 µg/Kg; AFG_1 and AFG_2: 2 µg/Kg; FB_1: 1 µg/Kg; FB_2: 2 µg/Kg; ZEA: 5 µg/Kg; OTA: 2 µg/Kg).

[b] LOQ: limit of quantification (for all mycotoxins: 5 µg/Kg except for ZEA: 10 µg/Kg)

[c] Aflatoxins: positivity for at least one aflatoxin among AFB_1, AFB_2, AFG_1 and AFG_2

[d] Fumonisins: positivity for at least one fumonisin among FB_1 and FB_2

Table 3

Concentrations of mycotoxins (µg/kg dry matter) in commercial extruded dry dog food.

	Standard dog foods			Premium dog foods			Regulatory limits (µg/kg) (2006/576/EC)
	Mean ± SD[a]	Median[b]	Max Val[c]	Mean ± SD[a]	Median[b]	Max Val[c]	
DON	103±75	99.4	281	81.3±61.7	57.7	246	5000[d]
FB_1	491±433[g]	416	1503	80.2±74.7[h]	59.0	325	
FB_2	113±93[g]	100	388	38.5±40.4[h]	24.4	155	
$FB_1 + FB_2$	599±507[g]	500	1746	103±99[h]	66.6	350	5000[e]
OTA	23.8±9.9[g]	21.7	41.1	13.0±9.7[h]	10.3	40.2	50[f]

DON, deoxynivalenol; FB_1, fumonisin B_1; FB_2, fumonisin B_2; OTA, ochratoxin A. The values for aflatoxins and zearalenone are not reported since lower than the corresponding limit of quantification in 88% and 75% of the samples, respectively.

[a] arithmetic mean ± standard deviation

[b] median of all positive samples

[c] maximum quantified value

[d] limit for generic complete or complementary feedingstuffs

[e] limit for pet animals

[f] limit for pigs

[g,h] Means within a row with different superscript letters differ ($P < 0.001$)

628 **Table 4**

629 Multi-residuals in 48 samples of commercial extruded dry dog foods (24 standard and 24
630 premium).

	Standard dog foods	Premium dog foods
1 mycotoxin	0	1
2 mycotoxins	3	6
3 mycotoxins	15	10
4 mycotoxins	6	6
5 mycotoxins	0	1

631